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Short-Range and Long-Range Guidance by Slit and Its Robo Receptors: Robo and Robo2 Play Distinct Roles in Midline Guidance

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Summary

Previous studies showed that Roundabout (Robo) in *Drosophila* is a repulsive axon guidance receptor that binds to Slit, a repellent secreted by midline glia. In *robo* mutants, growth cones cross and recross the midline, while, in *slit* mutants, growth cones enter the midline but fail to leave it. This difference suggests that Slit must have more than one receptor controlling midline guidance. In the absence of Robo, some other Slit receptor ensures that growth cones do not stay at the midline, even though they cross and recross it. Here we show that the *Drosophila* genome encodes three Robo receptors and that Robo and Robo2 have distinct functions, which together control repulsive axon guidance at the midline. The *robo,robo2* double mutant is largely identical to *slit*.

Introduction

Most growth cones in the developing central nervous system (CNS) confront and respond to signals from the midline. In *Drosophila*, the midline consists of a distinct set of specialized glia and neurons, which form a boundary separating the two mirror symmetric halves of the CNS. The midline glia secrete a number of factors, including Netrins and Slit, which play profound roles in patterning axon pathways and specific guidance decisions (reviewed in Tessier-Lavigne and Goodman, 1996).

The majority of CNS growth cones extend toward the midline. Most of these growth cones enter and cross the midline. Once they traverse and leave the midline, they never cross it again. Once across the midline, they change behavior and turn up or down in a specific longitudinal pathway on the other side. A minority of growth cones project on their own side of the midline, including some that never extend toward the midline and others that initially extend toward the midline and then abruptly stop and turn to extend up or down on their own side.

The *roundabout* (*robo*) and *commissureless* (*comm*) genes were identified in a large-scale mutant screen in *Drosophila* for genes that control the decision by axons to cross or not to cross the CNS midline (Seeger et al., 1993). In *robo* mutant embryos, too many axons cross and recross the midline. *robo* encodes an axon guidance receptor of the immunoglobulin superfamily (Kidd et al., 1998a) that is highly conserved in fruit flies, nematodes (Zallen et al., 1998), and mammals (Kidd et al., 1998a). For those axons that never cross the midline, Robo is expressed at high levels on their growth cones from the outset. For the majority of commissural axons that do cross the midline (but only once), Robo is expressed at high levels on their growth cones after they cross the midline. The combination of genetic analysis and expression data led to the conclusion that Robo was a repulsive guidance receptor for a midline repellent.

comm mutant embryos display the opposite phenotype to *robo*—no axons cross the midline. Comm is a novel transmembrane protein (Tear et al., 1996). Overexpression of Comm (i.e., the *comm* gain of function) leads to a phenotype similar in some respects to the *robo* loss of function (Kidd et al., 1998b); increasing Comm leads to a reduction of Robo levels. Comm appears to be a potent negative regulator of the Robo receptor on growth cones.

Subsequent experiments revealed that Slit is the midline repellent that functions as the Robo ligand (Kidd et al., 1999) and that this function is conserved across phylogeny (Brose et al., 1999). Slit is a large extracellular matrix protein secreted by midline glia (Rothberg et al., 1988, 1990). In *robo* mutant embryos, growth cones that normally do not cross the midline now do so. In *slit* mutant embryos, these same growth cones enter the midline but never leave it. Moreover, they continue to express high levels of Robo even while extending along the midline. *slit* and *robo* display dosage-sensitive genetic interactions, indicating that they function in the same pathway (Kidd et al., 1999). Biochemical analysis shows that Slit directly binds to Robo in *Drosophila* and mammals (Brose et al., 1999). Thus, Slit acts as a short-range repellent, and Robo functions as its receptor, to control axon crossing of the midline. In *Drosophila*, Slit is also required for migration of muscle precursors away from the midline; this function as a long-range chemorepellent also involves the Robo receptor (Bashaw and Goodman, 1999; Kidd et al., 1999).

In a *robo* mutant, axons cross and recross the midline, but they do not stay at the midline. Clearly, Robo plays a major role in controlling midline guidance. But Robo alone cannot control all aspects of the decision to cross or not to cross the midline, and Robo cannot be the only Slit receptor. If the midline, with its expression of Netrins (Serafini et al., 1994; Harris et al., 1996; Mitchell et al., 1996), is such an attractive place as an intermediate target, then why do growth cones ever leave the midline? Why don't these growth cones fasciculate with their contralateral homologs (which have reached the midline from the other side) and extend longitudinally together along the midline? This is in fact exactly what they do in a *slit* mutant in which all axon pathways collapse at the midline. Thus, in what appears to be the complete absence of the repulsive signal, axons are attracted to the midline and never leave it. The difference between the *robo* and *slit* mutant phenotypes is striking

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and suggests a missing component—a putative second Slit receptor.

Simply by observing the behavior of growth cones at and near the midline, we can describe two different kinds of repulsive functions controlled by Slit. On the one hand, Slit functions as a repulsive barrier that prevents growth cones expressing high levels of Robo from entering the midline. On the other hand, Slit functions as an "antilinger" repellent, which permits growth cones to enter and cross the midline but prevents them from staying there. These two different flavors of repellent activity are both mediated by the same ligand. One model would suggest that these two functions represent purely quantitative differences in the amount of repulsive signaling. Alternatively, these two repulsive functions might represent some qualitative difference. Determining how these different types of repulsion are generated requires identification and analysis of the second putative Slit receptor.

This other Slit receptor must also be able to be downregulated by Comm protein. Whereas moderate *comm* overexpression generates a *robo*-like phenotype by downregulating Robo protein (Kidd et al., 1998a), strong overexpression of *comm* results in a *slit*-like phenotype (Kidd et al., 1999), presumably by downregulating the expression of multiple Slit receptors.

Analysis of the *Drosophila* genome reveals two additional Robo receptors: Robo2 and Robo3. Either or both of these receptors are in principle good candidates to help control midline guidance. However, their different temporal and spatial patterns of expression point to Robo2 as the better candidate to contribute to midline guidance because it is expressed earlier and more broadly. In this paper we present evidence that the Robo and Robo2 receptors play unique and dynamic roles during midline guidance.

All three Robos have similar ectodomains and all three bind Slit. *robo2* and *robo3* lie close together on the second chromosome and are more closely related to one another than either of them is to *robo*. Their cytoplasmic domains diverge from Robo, and they lack several of the canonical conserved motifs found in *Drosophila* Robo and mammalian Robos, suggesting that Robo2 and Robo3 have different signaling capability than Robo. In particular, both Robo2 and Robo3 lack the binding site for Enabled, which is a major output of Robo (Bashaw et al., 2000).

Robo and Robo2 function during early stages of axon outgrowth to control midline guidance. As described in detail in our related paper (Simpson et al., 2000), Robo2 and Robo3 function during later stages to control lateral position. Because of its dual role, Robo2 has a highly dynamic pattern of expression, initially being expressed in all neurons (and growth cones) to prevent them from staying at the midline and later disappearing from some neurons and becoming restricted to only those growth cones that extend in lateral pathways.

The model that Robo and Robo2 together control midline guidance leads to a clear prediction—the double mutant combination of *robo* and *robo2* should generate a phenotype that resembles *slit*. A corollary of this model is that increasing Comm must also lead to a downregulation of Robo2. Both of these predictions are born out by the results presented here.

Results

Identification of Additional Robos: Cloning *robo2* and *robo3*

robo2 and *robo3* were initially detected by searching the *Drosophila* genomic database (Adams et al., 2000) for sequences similar to *robo*. Homology and protein prediction programs identified two paralogs of *robo*. cDNAs were obtained from the LD embryonic library (Rubin et al., 2000) and sequenced. The *robo2* and *robo3* genes are quite close to each other and facing in opposite directions on the left arm of chromosome 2 at location 22A; *robo* is located on the right arm of chromosome 2 at location 58F (Figure 1A).

The *robo2* cDNA encodes a 1540 amino acid protein with the same domain structure as Robo. The extracellular region contains five immunoglobulin-like (Ig) domains and three fibronectin type III (Fn) domains, followed by a single-pass transmembrane domain and a 450 amino acid cytoplasmic domain. The homology between Robo and Robo2 is highest in the extracellular region, ranging from 52%–53% (in the first two Ig domains) to 29% and dropping to less than 23% in the cytoplasmic region (Figure 1B). Robo2 lacks two of the four conserved cytoplasmic motifs that Robo shares with its orthologs in other species (Figure 1C) (Kidd et al., 1998a; Bashaw et al., 2000). The first two of these motifs (CCO and CC1), which are tyrosine phosphorylation sites (Bashaw et al., 2000), are maintained, but the second two motifs (CC2 and CC3), a proline-rich Enabled binding motif and another polyproline stretch, are missing. In Robo2, the proline-rich Enabled binding motif (CC2) is replaced by a polyglutamine repeat. Interestingly, although several of the mammalian Robo orthologs contain all four conserved cytoplasmic motifs, a more divergent Robo family member, Rig-1, appears to lack CC1 (Yuan et al., 1999).

Robo3 resembles Robo2 more closely than it does Robo; it too lacks two of the four cytoplasmic motifs (Figure 1C). The genomic organization of *robo2* and *robo3* is very similar. Both have large first introns (23 and 17 kb, respectively), while the first intron in *robo* is less than 1 kb. The intron and exon sizes are similar, and which exons code for particular domains is also conserved, suggesting that *robo2* and *robo3* may be the result of a recent duplication.

The complete *Drosophila* genome reveals no other candidate Robos. Examination of the vertebrate databases shows that the identified homologs have cytoplasmic domain organization more like *Drosophila* Robo than like Robo2 or Robo3, indicating that Robo is closer to the common ancestor with vertebrates. Robo2 and 3 resemble each other and Robo more closely than they resemble any of the mammalian Robos or the *Caenorhabditis elegans* Sax3. Examination of the exon–intron boundaries within the coding regions of the three *robo* genes suggests that *robo2* and *robo3* may be the result of a recent duplication event (Figure 1D). This phylogeny holds when the complete protein sequences of the homologs are compared, as well as when the extracellular domains are aligned and when only the most highly conserved first Ig domain is used. The Robo receptor family is related to other neural adhesion and guidance

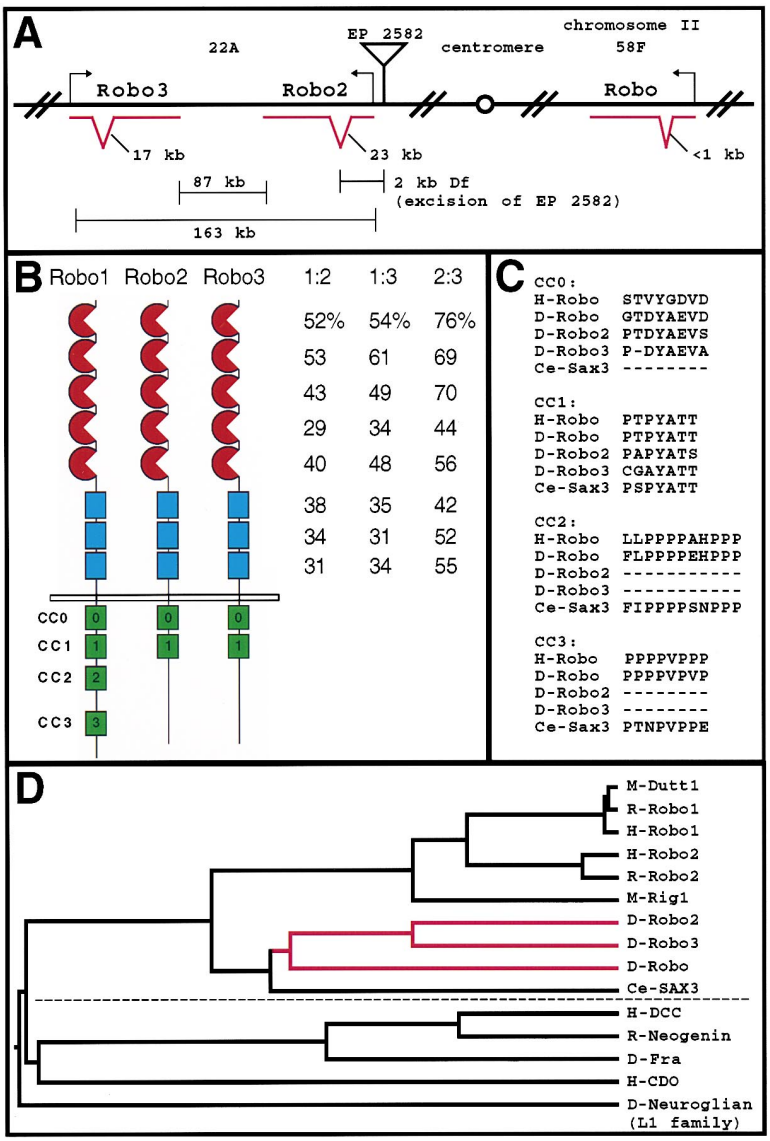

Figure 1. Genomic Organization, Homology, and Family Tree of *Drosophila* Robo Receptors

(A) The genomic positions of Robo, Robo2, and Robo3 are shown on a schematic representation of chromosome 2. Robo2 and 3 are 87 kb apart and transcribed in opposite directions. The position of the P element (EP 2582) that can overexpress Robo2 and that was excised to generate Robo2 mutations is shown in the first exon of Robo2, ~100 bp upstream of the presumptive translational start. The sizes of the first introns and the extent of the Robo2 excisions are noted.

(B) The Robo receptors share a common extracellular domain structure of five immunoglobulin domains and three Fibronectin type III domains. The percent homology between analogous domains of different *Drosophila* Robos is given at the right. Intracellularly, Robo2 and Robo3 have only the conserved motifs CC0 and CC1.

(C) Sequences of the conserved cytoplasmic motifs in the Robo homologs. In Robo, CC0 and CC1 can be phosphorylated by Abl. CC2 is an Enabled binding motif.

(D) A clade generated by comparing the full-length sequences of Robo, Robo2, and Robo3 to their closest relatives in the genetic databases illustrates that Robos form a distinct subfamily of the Ig superfamily and that the mammalian and *C. elegans* homologs resemble Robo more than they resemble Robo2 or Robo3.

molecules like DCC/Frazzled and Neuroglian but is a distinct subgroup of the Ig superfamily (Figure 1D).

Expression of Robo2 and Robo3

In situ hybridization and immunocytochemistry studies show that all three *robos* are expressed in the embryonic CNS during the period of axon outgrowth. *robo* expression begins first at embryonic stage 10. *robo2* expression is first visible at stage 11 and becomes restricted to a smaller subset of neurons later in development by stage 15 (Figures 2D and 2E). *robo3* expression does not begin until late stage 13 and is limited to fewer neurons.

Comparing the cells that express *robo*, *robo2*, and *robo3* (Figure 2) gives clues about the potential roles the three different Robo receptors might play during axon guidance in terms of two different events. On the one hand, they function during the early establishment of midline crossing decisions (as described here). On the other hand, they appear to function during the later establishment of lateral position (i.e., the location and choice of specific longitudinal axon pathways in the medial-lateral axis; see Simpson et al., 2000). Staining in situ–labeled embryos with an antibody against Fasciclin II (mAb 1D4) and examining the preparations with Nomarski optics allows us to identify some of the individual neurons that express particular Robo family members. Previous studies determined the pattern of growth of a number of key pioneer neurons and determined how they ultimately establish two of the major longitudinal axon pathways in the CNS (Goodman and Doe, 1993; Hidalgo and Brand, 1997).

robo mRNA is present at approximately equal levels in most of the neurons of the early CNS, including pCC, aCC, MP1, dMP2, and vMP2 (Figures 2A and 2D). This is in agreement with previous studies (Kidd et al., 1998a), which showed that most if not all CNS neurons express *robo* mRNA from the onset of axon outgrowth. This observation has led to the conclusion that it is some form of posttranscriptional regulation that controls the

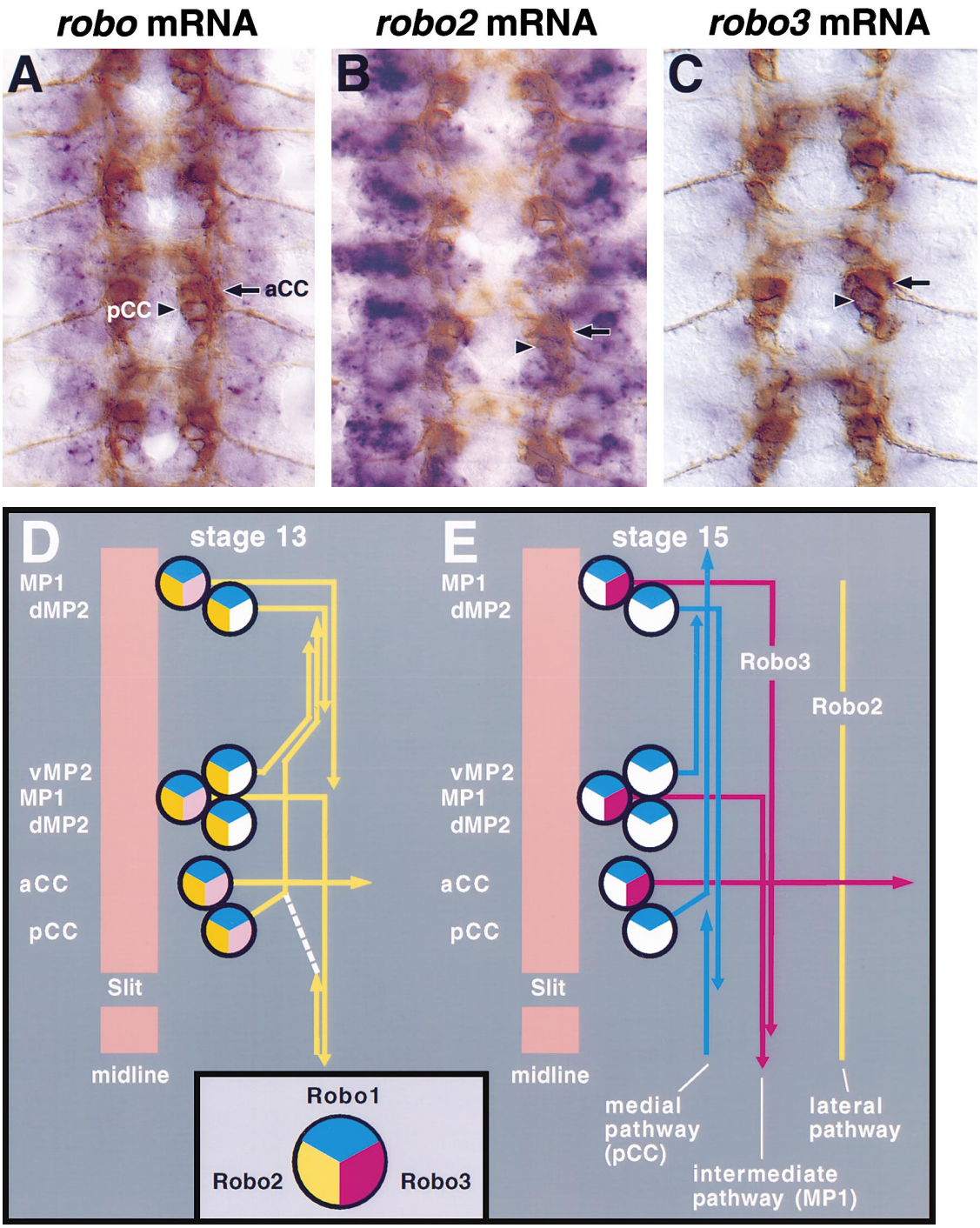

Figure 2. Expression of Robo Family Genes

In situ hybridization analysis shows that *robo* (A), *robo2* (B), and *robo3*(C) mRNA transcripts are present in the embryonic stage 13 central nervous system and that their expression patterns are different. The brown HRP staining shows anti-Fas II (mAb 1D4) expression for identification of neurons. The diagram below schematizes the expression of the Robos at stage 13 (D) and stage 14 (E) as deduced from their mRNA expression. Robo and Robo2 are expressed in many neurons, including aCC and pCC, while Robo3 is expressed there only at a low level. Later, Robo2 expression is shut off in these neurons, and Robo3 expression is elevated, especially in MP1 and aCC.

striking pattern of Robo protein expression in which the protein dramatically increases on growth cones after they cross the midline.

robo2 RNA can be detected in the aCC and pCC neurons at early stage 13. The expression level of *robo2* in these cells increases throughout stage 13 (Figures 2B and 2D) and then vanishes during stage 14 (Figure 2E). *robo2* is transiently expressed in a variety of other pioneer neurons in the CNS, including MP1, dMP2, and vMP2. All of these growth cones normally project ipsilaterally without crossing the midline.

The four axons from pCC, vMP2, MP1, and dMP2

initially selectively fasciculate as they extend in a pairwise fashion and transiently display a high affinity for one another; they all express high levels of Fas II (Goodman and Doe, 1993; Lin et al., 1994) (Figure 2D). However, they subsequently selectively defasciculate as pCC and vMP2 pioneer the medial Fas II pathway, while MP1 ultimately pioneers the intermediate Fas II pathway (Hidalgo and Brand, 1997). The defasciculation of these axons and their separation to form these two distinct longitudinal pathways occurs when *robo2* expression in all of these neurons declines (Figure 2D versus 2E); this is the same period when *robo3* appears in a subset of these neurons.

robo3 is expressed later than *robo2* and in a highly restricted subset of CNS neurons. *robo3* is not expressed at early or midstage 13 but, by late stage 13, begins to be expressed in MP1 (which pioneers the intermediate Fas II pathway) and aCC (which is a motoneuron that exits the CNS and extends into the periphery). *robo3* expression increases throughout stage 14 in both MP1 and aCC. *robo3* mRNA is not detected in pCC, vMP2, or dMP2.

The pCC, vMP2, MP1, and dMP2 growth cones pioneer the first two longitudinal axon pathways. All four growth cones initially extend right next to the midline but normally do not cross it. In a *robo* mutant, all four growth cones cross and recross the midline (Seeger et al., 1993; Kidd et al., 1998a, 1998b). In a *slit* mutant, all four growth cones enter the midline and do not leave it (Kidd et al., 1999). From the beginning of axon outgrowth, *robo* is expressed in all four neurons. Similarly, *robo2* is transiently expressed in all four neurons by early stage 13. However, it is not until late stage 13 that *robo3* is expressed at low levels in two of these four neurons. Thus, *robo* and *robo2* are expressed early enough in these ipsilaterally projecting pioneer neurons to prevent them from entering or crossing the midline, whereas *robo3* is not. As *robo3* expression begins, *robo2* expression becomes more restricted. As development proceeds, both *robo2* and *robo3* expression becomes restricted to a pattern that specifies the lateral position of axons (Simpson et al., 2000).

Antibody staining using monoclonal and polyclonal antisera raised (in mouse) against the three different Robos supports the mRNA expression data. Robo and Robo2 proteins appear earlier than Robo3 and, in general, appear to be expressed on many if not all of the early ipsilaterally projecting axons. Later in development, as Robo3 protein appears, the patterns of expression resolve into a restricted pattern for Robo2 and Robo3. Robo, Robo2, and Robo3 are found on the longitudinal tracts of the CNS scaffold but not in the commissural segments of contralaterally projecting axons. All three Robos are expressed on growth cones as revealed by immunoelectron microscopic analysis (Simpson et al., 2000). Robo is present across the entire medial-lateral span of the longitudinal pathways, while Robo3 is expressed on axons in the lateral two thirds, and Robo2 is further restricted to the lateral third only of the longitudinal axon pathways (for details and photographs, see Simpson et al., 2000). Immunocytochemistry also shows that the Robo2 protein is found in the heart, the early trachea, and the lateral body wall muscles, where it subsequently resolves to the muscle attachment sites. A further analysis of the role of Robo family members in heart and muscle patterning is in progress (S. Kramer and C. S. G., unpublished data).

Generation of *robo2* Mutants

Loss of the Robo receptor results in a phenotype in which certain axons, particularly those that normally extend in medial axon pathways, ectopically cross and recross the midline. The absence of Slit, the Robo ligand, results in a collapsed midline in which all axons extend toward the midline and then fail to leave it. The most straightforward explanation for why the loss-of-function phenotypes of the Slit ligand and the Robo receptor differ is that there is another repulsive receptor responding to Slit. Given the expression data described above, Robo2 is the obvious candidate. Below, we describe the generation and analysis of *robo2* mutants. However, we also tested the role of *robo3* in midline guidance, using dsRNA (the RNAi method) to eliminate function. Because Robo3 plays a major role in lateral position but not in midline crossing per se the *robo3* analysis can be found in our related paper (Simpson et al., 2000).

To determine if Robo2 has an essential function, whether it plays a role in midline guidance, and, in particular, whether its presence drives axons to leave the midline in *robo* mutants, mutations in *robo2* were generated. We identified a P element transposon from the Rørth EP collection (Rørth, 1996) inserted immediately upstream of the *robo2* signal sequence and mobilized it to generate imprecise excisions that lead to small deletions in the *robo2* coding region. These mutations are lethal and show a central nervous system phenotype that reveals Robo2's contribution to axon guidance.

Four excisions were molecularly characterized; all result in deletions of 1–2 kb that remove the first exon of *robo2,* which includes the translation start site and the signal sequence, and terminate in the large first intron. These mutants fail to complement each other and are lethal when crossed to deficiencies for the region. The excision mutants retain a very low level of *robo2* mRNA (and a little bit of protein immunoreactivity) but appear to behave as genetic nulls. The various mutant alleles and the mutant/deficiency combinations have qualitatively similar phenotypes, although, as described below, certain quantitative aspects can change. Injection of *robo2* dsRNA into wild-type embryos replicates the *robo2* mutant phenotype, but injection into the *robo2* excisions did not substantially worsen the *robo2* phenotype, supporting the classification of these excisions as null or nearly null alleles.

When examined with mAb BP102 against all CNS axons, the *robo2* mutant looks slightly abnormal but much closer to wild-type than does the *robo* mutant (Figures 3B and 3C). This is presumably why *robo2* mutants were not identified in the initial midline mutant screen of the genome, which used mAb BP102 (Seeger et al., 1993).

When examined with mAb 1D4 against Fas II, clear mutant phenotypes are revealed (Figure 4C). In the *robo2* mutant, some axons ectopically cross the midline. This ectopic crossing phenotype is much weaker and less penetrant than in the *robo* mutant. In the *robo2* mutant there is disorganization of the longitudinal tracts. At stage 16, Fas II is normally expressed on four major longitudinal axon pathways (out of a total of 20 or more),

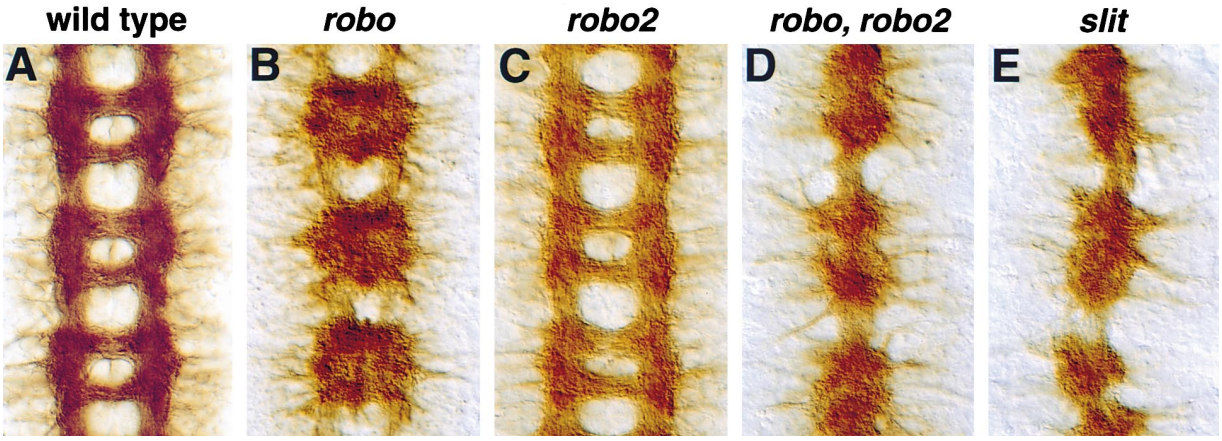

Figure 3. The *robo,robo2* Double Mutant Phenotype Looks Like *slit*

Fillet preparations of the *Drosophila* embryonic central nervous system (Stage 16; anterior up) stained with mAb BP102, a monoclonal antibody that stains all CNS axons (A), demonstrates that loss of *robo* (B) causes severe ectopic midline crossing of the midline, thickening of the commissures, and reduction in the longitudinal connectives between segments. *robo2* mutants (C) look relatively normal when stained with BP102. Embryos mutant for both *robo* and *robo2* (D) show a compressed midline where all the axons approach the midline and cannot leave. This phenotype is identical to that generated by loss of *slit* (E), the repulsive ligand for Robo receptor family members.

of which three are clearly visible in a single optical focal plane and are diagnostic for lateral positioning. One of the Fas II pathways (the pCC pathway) is medial, another is intermediate (the MP1 pathway), and a third is lateral (this one is the last to form). The fourth Fas II pathway is more ventral directly below the medial Fas II pathway and will not be further mentioned.

The disorganization of the Fas II pathways appears as "braiding," since, instead of maintaining their parallel alignment (i.e., medial, intermediate, and lateral), the three diagnostic Fas II bundles on each side of the CNS now cross over and intermittently join with each other on their own side. Segments that show misrouting of axons between bundles on the same side of the midline are more common than those that show axons crossing the midline.

The frequency of aberrations is higher in the excision/deficiency embryos as compared to the excision/excision embryos, but this may be due to the fact that the deficiency removes a number of genes in addition to *robo2*—notably *robo3*. Heterozygosity for one *robo* can enhance the null phenotype of another; *robo2* dominantly enhances a *robo* mutation (Figure 4F). Thus, it is plausible that the increase in *robo2* defects in the excision/deficiency combination is due to heterozygosity for *robo3* rather than to any additional reduction in Robo2.

The *robo2* phenotype can also be visualized using anti-Connectin mAb (Meadows et al., 1994). Connectin is a cell adhesion molecule that is expressed in the CNS by a subset of axons that fasciculate in two longitudinal axon pathways, one medial and the other intermediate to lateral (Figure 4H; Nose et al., 1992). Some of these axons cross in the anterior commissure, where they also express Connectin. In *robo2* mutants, the two Connectin pathways are often fused together into a single group of axons (Figure 4J). The Fas II and Connectin staining patterns suggest that the loss of function of *robo2* affects the ability of these axons to locate their correct lateral position and to form their correct pattern of longitudinal axon pathways (this is the focus of our related paper, Simpson et al., 2000). *robo* mutants, on the other hand, still show two Connectin pathways, but axons in the medial of the two Connectin pathways appear to ectopically cross the midline (just as the medial Fas II axons abnormally cross the midline).

robo,robo2 Double Mutants Have the *slit* Phenotype

The ectopic crossing of axons in *robo2* mutants indicates that Robo2 does indeed contribute to midline guidance as well as to lateral position. To determine if Robo2 supplies the repulsive force that drives axons to leave the midline in *robo* mutants, *robo,robo2* double mutants were generated by recombination. The *robo, robo2* double mutants were examined with mAbs 1D4 and BP102 and found to be phenotypically identical to *slit* (Figures 3D, 3E, 4D, and 4E). All axons are initially attracted to the midline (presumably guided in part by Netrins). But once these axons enter the midline, they are unable to leave. In a *robo* mutant alone, the axons leave the midline but recross it. In the double mutant, they never leave the midline, just as in a *slit* mutant. Thus, Robo and Robo2 together can account for all of the function of Slit in midline guidance. In the absence of Robo, it is the small amount of Robo2 on the growth cones that drives them to leave the midline, even though they can cross and recross the midline.

The relative contribution of Robo and Robo2 to prevention of crossing can be clarified by examining their ability to dominantly enhance each other (i.e., the phenotype generated by removing 100% of one protein is enhanced by removing 50% of the other protein). Heterozygosity for *robo* in a *robo2* null background ($robo^{+/-}$ $robo2^{-/-}$) increases the midline disruption (Figure 4G). These embryos show a dramatic increase in ectopic midline crossing as compared to *robo2* mutants alone, and the crossing involves all three of the Fas II longitudi-

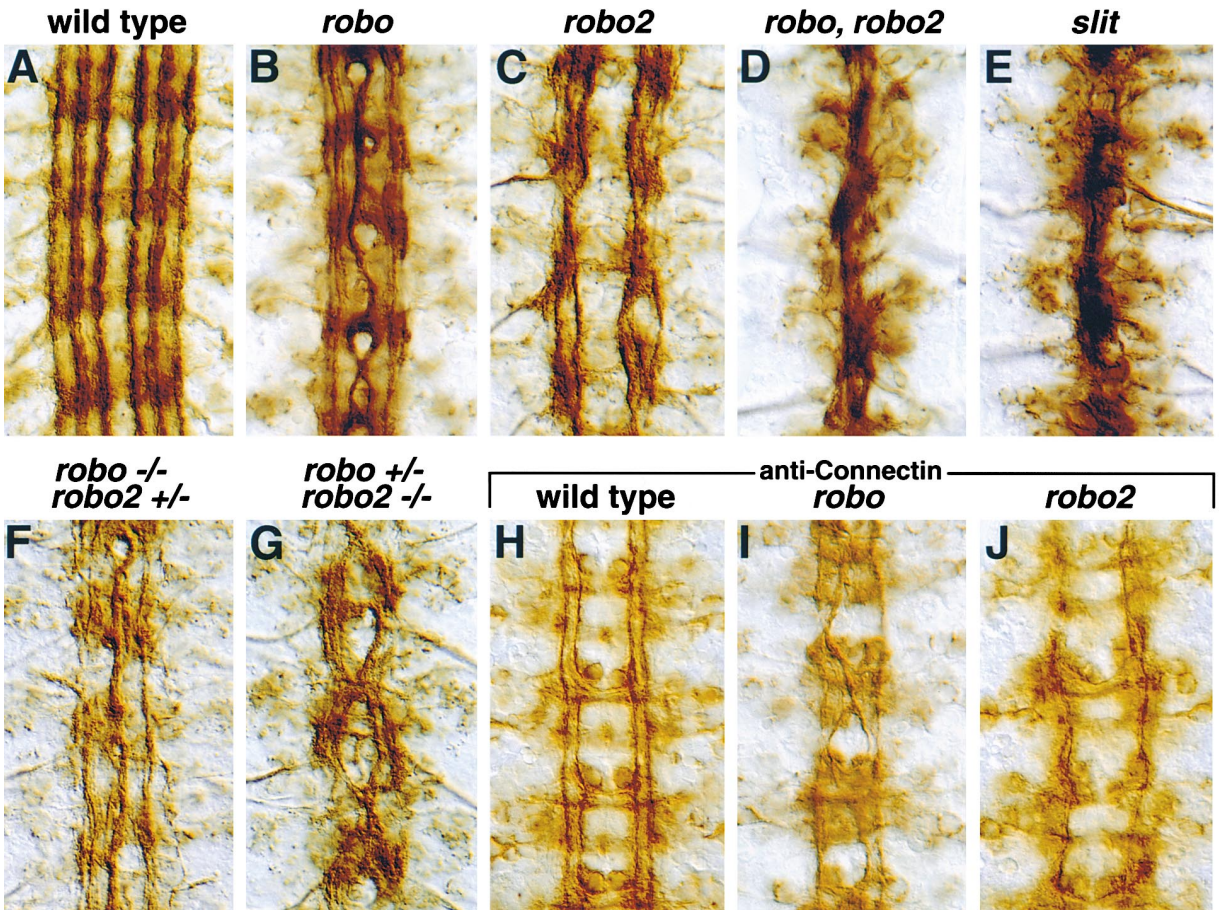

Figure 4. The *robo* and *robo2* Phenotypes Are Distinct

The axon scaffold of stage 16 embryos (fillet preparation; anterior up), when stained with mAb 1D4, an antibody to Fasciclin II, shows three longitudinal fascicles (out of many) on each side of the midline (A). These three fascicles are referred to as the medial, intermediate, and lateral Fas II pathways. (There is a continuous set of axons on each side of the midline—the spaces between the Fas II bundles are full of axons that do not express Fas II. There are also additional Fas II pathways out of the plane of focus shown in these pictures.) mAb 1D4 is a useful marker for a subset of axons because it reveals more subtle misrouting phenotypes that are invisible with mAb BP102 that stains the entire axon scaffold (see Figure 3). In *robo* mutants (B), the medial Fas II pathway (closest to the midline) ectopically crosses and recrosses the midline. The intermediate and lateral fascicles remain on their own side and are largely wild type in appearance. In *robo2* mutants (C), the Fas II axons stay on their own side except for occasional crossovers, but the organization of the longitudinal tracts is disrupted. There are crossovers between intermediate and medial fascicles and between the lateral and intermediate fascicles on the same side of the midline. This phenotype is called "braiding" and is present in all of the *robo2* allelic combinations in the *robo2* RNAi injections and in the *robo2*-deficiency embryos as well. The *robo,robo2* double mutant (D) and the *slit* mutant (E) embryos have all of the Fas II pathways collapsed together and running along the midline. The axons start off at their normal positions and extend toward the midline, but, because the Slit-Robo repulsion system is absent in these genotypes, the axons fail to leave the midline. *robo* and *robo2* can dominantly enhance each other. A *robo* mutant heterozygous for *robo2* (F) shows ectopic crossing of the medial Fas II pathway like a *robo* mutant, but, in many segments, the medial pathway collapses entirely onto the midline in a phenotype resembling *slit*. In a *robo2* mutant, loss of a single copy of *robo* causes much more ectopic crossing than is seen in the *robo2* mutant alone (G). These crossovers now include the intermediate and lateral fascicle as well as the medial one. Another antibody that marks a subset of axons is anti-Connectin. There are two bundles of axons expressing Connectin on each side of the midline in a wild-type stage 16 embryo (H). *robo* mutants stained with anti-Connectin (I) show ectopic axon crossing, but, in most segments, two distinct fascicles are still visible on each side of the midline (from Kidd et al., 1998b). In contrast, *robo2* mutants (J) show little ectopic midline crossing, but only one fascicle is now visible on each side. The absence of *robo2* has a more profound affect on the organization of the parallel longitudinals than does the loss of *robo*, while *robo* shows more inappropriate crossing than does *robo2*.

nal pathways (not just the medial Fas II pathway, as seen in *robo* mutants alone). Thus, one copy of *robo* (presumably producing 50% of protein) is not sufficient to prevent crossing, but it is sufficient to prevent axons from lingering at the midline in the absence of *robo2*.

Heterozygosity for *robo2* in a *robo* null background ($robo^{-/-}robo2^{+/-}$) leads to a different enhancement in the midline phenotype (Figure 4F). Just as in a *robo* mutant, so too in a $robo^{-/-}robo2^{+/-}$ mutant; it is only the axons in the medial Fas II pathway that ectopically enter and cross the midline. However, this subset of axons usually does not leave the midline, and, instead, the two medial Fas II pathways fuse and run along the midline. (In a *slit* mutant—or *robo,robo2* double homozygous mutant—all three Fas II pathways are fused along the midline.) Thus, whereas one copy of *robo* (in the absence

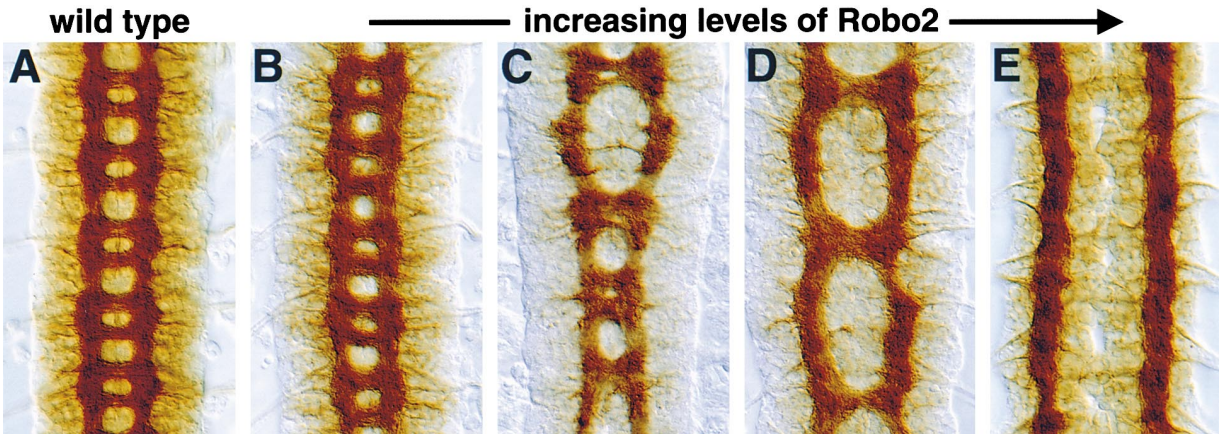

Figure 5. Overexpression of Robo2 Leads to a Biphasic Range of Phenotypes

Using an EP P element insertion upstream of *robo2* and different transgenic inserts of *UAS-robo2* and *elav-GAL4* driver stocks of various strengths, an expression series of increasing levels of Robo2 protein was generated. The wild-type axon scaffold stained with mAb BP102 is shown in (A). A low level of Robo2 overexpression (B) has very slight effect on the appearance of the scaffold—some axons ectopically cross, resulting in a mild *robo* phenotype. Increasing the level of Robo2 gives a stronger *robo*-like phenotype in some segments, while other segments appear like *commissureless*, lacking all midline crossing (C). In still higher levels, extra Robo2 in all neurons results in an almost complete (D) to complete (E) *commissureless*-like CNS.

of *robo2*) is sufficient to prevent axons from staying at the midline, one copy of *robo2* (in the absence of *robo*) is not.

Robo and Robo2 also cooperate in other developmental processes. Slit, Robo, and Robo2 function during mesoderm migration. After gastrulation in *Drosophila*, many myoblasts migrate laterally away from the ventral midline. In *slit* mutant embryos, some mesoderm cells do not migrate away from the midline and, instead, form muscles abnormally near the midline that often stretch across the midline (Kidd et al., 1999). A weak version of this phenotype is observed in the *robo* mutant, suggesting that it alone cannot control mesoderm migration away from Slit. A similarly weak phenotype is observed in the *robo2* mutant. However, a strong phenotype is observed in the *robo,robo2* double mutant. This phenotype is very similar to the *slit* phenotype; many mesodermal cells do not migrate away from the midline, and, instead, some developing muscles are found ectopically crossing the midline. Thus, Robo and Robo2 appear to cooperate in controlling mesoderm migrations away from the midline. Robo and Robo2 also appear to cooperate in governing proper cell migrations and alignment of cardioblasts in the embryonic heart and in the further development of muscle, including the identification of proper insertion sites (S. Kramer, personal communication).

Slit Is the Ligand for Robo2 and Robo3

The extracellular sequence similarity between Robo, Robo2, and Robo3 and the *robo,robo2* double mutant phenotype, strongly suggests that Robo2 and Robo3 also bind Slit. This was tested in cell culture. Full-length and the N-terminal cleavage fragment of Slit remain predominantly cell associated when trangenically expressed in culture (Brose et al., 1999). AP-tagged Robo2 and Robo3 ectodomains bind to Slit-expressing COS cells but not mock-transfected or untransfected cells (data not shown). The relative affinities of the receptors for Slit were examined using equilibrium binding experiments in which Slit-expressing COS cells were overlaid with various concentrations of AP-tagged ectodomains of the various Robo receptors. Total AP activity bound to cells after washing was measured colorimetrically, and nonspecific binding to mock-transfected cells was subtracted. The analysis of quantitative differences among the three Robo receptors will be presented in a later paper. All have dissocation constants (Kds) in the range of 10–40 nM.

Increased Expression of Robo2

Overexpression of *robo2* demonstrates that Robo2 can act as a repulsive axon guidance receptor. Moreover, it reveals an important difference between Robo and Robo2. The UAS-GAL4 system (Brand and Perrimon, 1993) was used to drive *robo2* expression in all neurons in the embryonic CNS. An expression series of increasing levels of Robo2 was generated by using *elav-GAL4* driver stocks and *robo2* reporter stocks of various strengths. These included an EP P element insertion upstream of *robo2* (Rorth, 1996) and different transgenic inserts of *UAS-robo2*.

We observed a characteristic phenotypic series based on increasing levels of Robo2 that is different from what is seen with Robo (Kidd et al., 1998a, 1998b, 1999; Bashaw and Goodman, 1999; Bashaw et al., 2000). At the high end of expression levels, both genes generate a commissureless-like phenotype in which no axons cross the midline (Figure 5E). However, previous studies showed that increasing Robo expression led to a simple phenotypic series of increasing severity of the commissureless phenotype. Interestingly, something quite different is observed with Robo2.

A low level of Robo2 overexpression results in inappropriate midline crossing reminiscent of a partial *robo* loss-of-function phenotype (Figure 5B) and, with in-

Table 1. Suppression of the Robo2 Gain-of-Function Phenotype

Partial Genotype	Segments Scored	Commissures				
		Absent	Thin	Normal	Thick	Abnormal
EProbo2;elav-GAL4(3A)	178	48%	26%	5%	19%	2%
EProbo2;elav-GAL4(3A) ena^{GC1}/+	112	13%	27%	19%	37%	3%
EProbo2;elav-GAL4(3A) robo^{1}/+	166	3%	25%	9%	32%	3%
EProbo2;elav-GAL4(3A) slit^{2}/+	229	10%	7%	8%	69%	7%
EProbo2;elav-GAL4(3A) robo^{5}/robo^{1}	241	21%	20%	15%	40%	3%
UAS-robo;elav-GAL4(3A) robo^{5}/robo^{1}	178	0%	0%	69%	30%	0%

Expression of *robo2* at high levels in all CNS neurons generates a robust *commissureless*-like phenotype wherein no axons that stain with mAb BP102 cross the midline. Removing copies of other genes that potentially participate in the repulsion of axons from the midline can shift the *commissureless*-like phenotype toward a *robo*-like phenotype seen when *robo2* is misexpressed at lower levels or toward wild type when *robo2* is expressed normally in a subset of CNS axons. Loss of a copy of *slit*, *robo*, or *enabled* in a *robo2* overexpression stock shifts the number of segments with no commissures from approximately 50% to approximately 10%. Stage 15 and stage 16 embryos stained with mAb BP102 were scored.

creasing levels of Robo2, of a complete loss of function of *robo* (some segments in Figure 5C). As levels of Robo2 continue to increase, the response becomes biphasic (Figure 5C). The proclivity to cross the midline (and thus mimic the *robo* loss of function) is replaced at higher levels of Robo2 by an increasing tendency to avoid the midline (and thus mimic the *robo* gain of function) (one segment in Figure 5C, most segments in 5D, and all segments in 5E).

This biphasic phenotypic series with increasing levels of Robo2 is different from what is observed with Robo and suggests two opposing functions with different thresholds. On the one hand, moderate levels of Robo2 appear to be able to interfere with midline repulsion. One interpretation is that Robo2 disrupts Robo signaling, either by competing for Slit binding or by decreasing Robo's output strength. We find (see below) that Robo2 is capable of heterodimerizing with Robo (as well as both receptors being capable of homodimerizing). If the heterodimer has a weaker repulsive output than a Robo homodimer, then this could explain the decrease in midline repulsion at low increased levels of Robo2.

However, Robo2 does not just interfere with midline repulsion; it can also mediate it. Higher levels of ectopic Robo2 lead to the opposite phenotype in which axons fail to cross the midline. Evidently, Robo2 does have a repulsive output, just not as strong as that of Robo. Sufficient levels of Robo2 are capable of generating a complete commissureless phenotype (Figure 5E). Thus, at low levels, Robo2 decreases the strength of Robo signaling and permits inappropriate midline crossing, while, at higher levels, Robo2 is capable of mediating sufficient repulsive signaling to prevent midline crossing entirely.

The commissureless phenotype observed at the higher levels of Robo2 overexpression can be partially genetically suppressed by heterozygosity (i.e., removing one copy) of *robo*, *slit*, or *enabled* (Table 1). Although the number of commissures that form in these backgrounds is increased, the phenotype is more complex than simple suppression because in many cases the crossovers that now occur are inappropriate.

Adding a *robo* dominant-negative transgene (truncated just after the transmembrane domain) changes the phenotype at all levels of Robo2. The Robo dominant negative (roboDN) increases the ectopic crossing seen at low levels of Robo2 overexpression, and it causes ectopic crossing at higher levels of Robo2 overexpression as well. Whether this is suppression by interference with Robo2 repulsion directly or, alternatively, whether it results from cumulative loss of repulsion by reducing the efficacy of the Robo pathway is unclear. However, increasing levels of RoboDN in a wild-type background only look like a *robo* loss of function, no matter how much RoboDN is added, and not like a *robo,robo2* double mutant or *slit* mutant. This suggests that the RoboDN affects Robo output and not Robo2 output, making the second alternative above seem more likely.

Robo2 Can Homodimerize and Heterodimerize with Robo

Ectopic expression of low levels of Robo2 by all neurons causes ectopic crossing of axons reminiscent of a *robo* mutant. A possible explanation is that small amounts of Robo2 can interfere with repulsion by Robo. Perhaps Robo2, which lacks some of the conserved motifs found in the Robo cytoplasmic domain, has a less robust repulsive output than Robo. Extra Robo2 could interfere with Robo by dimerizing with it and creating a weaker receptor. Alternatively, Robo2 might interfere by competing for Slit binding or by sequestering downstream signaling components needed by Robo. In vitro analysis shows that the cytoplasmic domains of Robo2 and Robo can bind to one another (and homodimerize), suggesting that the interference might be direct.

The in vitro translated cytoplasmic domains of Robo and Robo2 can bind to GST-fusion proteins containing the cytoplasmic domain of Robo or Robo2. The homodimeric interactions are favored over the heterodimer by ~4-fold (data not shown). The binding of Robo to Robo2 and of Robo to itself is not altered in GST-Robo fusion proteins individually lacking conserved motif CC1, 2, or 3, nor in one lacking the 67 amino acids closest to the transmembrane domain. Further experiments to determine which cytoplasmic domains are sufficient and necessary for in vitro Robo and Robo2 dimerization are in progress.

Although Robo and Robo2 can interact in vitro, it is not known if they heterodimerize in vivo. They are coexpressed in certain cells and thus have the opportunity to function cooperatively, but they can clearly function independently, presumably as homodimers. Robo can

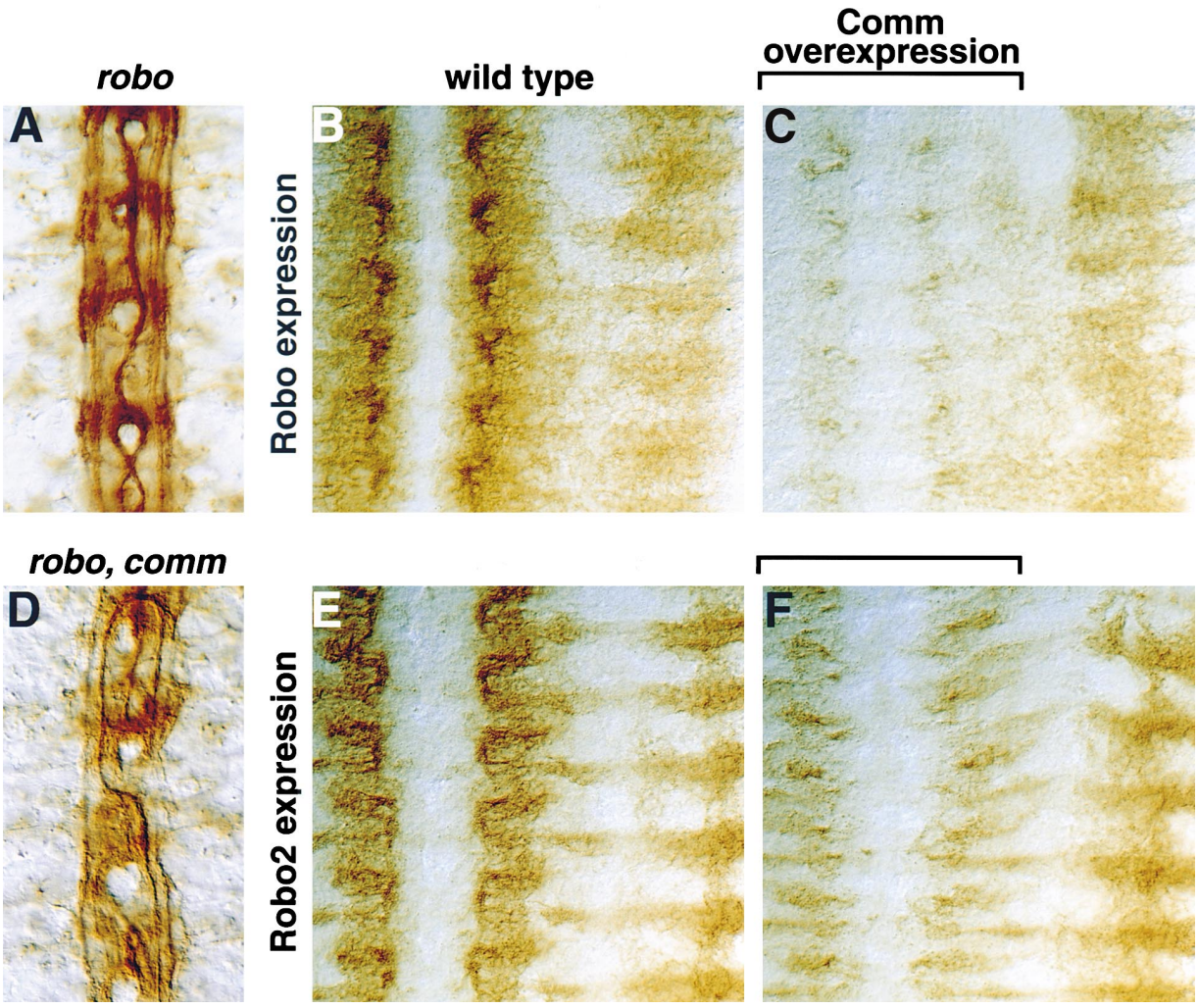

Figure 6. Commissureless Affects More Than Robo and Can Reduce the Levels of Robo2

In *robo* mutants, the medial Fas II pathway ectopically crosses the midline, while the intermediate and lateral Fas II pathways behave normally (A). In *robo;comm* double mutants, however, the intermediate pathway can also ectopically cross the midline, and there are occasional abnormalities in the lateral pathway as well (D). In some segments, the medial pathway travels along the midline, and, in others, several pathways appear to fasciculate together before crossing the midline inappropriately. The double mutant phenotype is somewhat variable, but the fact that the *robo* phenotype and the *robo;comm* phenotype are different suggests that Comm is doing another job in addition to downregulating Robo protein on commissural axons. A comparison of Robo protein levels at the midline in wild-type stage 12 embryos (B) and in embryos expressing extra Comm in the midline glia and CNS axons (C) (*UAS-comm* and *Sca-GAL4*) shows that adding Comm reduces the amount of Robo protein present in the CNS. The level of staining is constant; the two embryos shown were siblings stained in the same vial, and the level of staining in the tracheal precursors, far away from the source of Comm expression, is comparable. The identical experiment was performed to examine Robo2 protein levels. In wild-type stage 12 embryos (E), Robo2 is strongly expressed in some CNS neurons, but, when extra Comm is added (F), the level of Robo2 protein is markedly decreased in the midline near the extra Comm but not farther away in the body wall.

maintain a relatively normal CNS scaffold in the absence of Robo2. Robo2 can prevent the medial and lateral pathways from crossing the midline and all axons from lingering at the midline, in the absence of Robo. Although heterodimers have not yet been detected in vivo due to problems with coimmunoprecipitation sensitivity in whole-embryo preparations, the genetic results described above (i.e., the biphasic phenotypic series with increasing levels of Robo2) are consistent with this possibility.

Increasing Levels of Comm Downregulate Robo2 as Well as Robo

Commissureless protein can downregulate Robo2 as well as Robo. *comm* overexpression in midline glia and early neurons using *Scabrous-GAL4* can reduce the level of Robo2 protein in CNS axons just as it reduces the levels of Robo (Figures 6B, 6C, 6E, and 6F; Kidd et al., 1998b).

In *comm* gain-of-function embryos, the phenotype is *robo* like, but there is more disorganization of the outer (i.e., intermediate and lateral) pathways, presumably because Comm is downregulating Robo2 as well as Robo. In *comm* null mutants, Robo2 is still localized to the lateral pathways of the CNS scaffold (and Robo3 to the intermediate and lateral pathways), indicating that Comm is not required for the lateral restriction of Robo2 and Robo3. This restriction of Robo2 and Robo3 to specific subsets of neurons appears to be largely transcrip-

tional as revealed by in situ hybridization (see earlier section).

In contrast, the dramatic increase of Robo protein levels as growth cones cross the midline is, at least in part, regulated by Comm. The distinction is as follows: *which* neurons express any particular Robo family member (or combination of Robos) appears to be largely transcriptionally controlled, whereas *when* a given neuron displays on its axons any particular Robo family member (after the onset of transcription) appears to be controlled by other mechanisms, including Comm. Moreover, *where* a neuron expresses any particular Robo family member (i.e., the commissural versus longitudinal axon segment) also appears to be controlled by other mechanisms.

The *comm* gain of function shows that Comm can downregulate both Robo and Robo2. But does it normally regulate more than just Robo? In the original midline mutant screen paper (Seeger et al., 1993), the *robo;comm* double mutant was described as looking just like *robo* when stained with mAb BP102 (which labels all CNS axons). If the double mutant was indeed indistinguishable from *robo* alone, then this would suggest that Comm normally only regulates Robo. But this is not the case; distinct differences are observed when we compare the double (*robo;comm*) mutant with *robo* alone, using mAb 1D4 to stain the three major Fas II pathways (Figures 6A and 6D).

In a *robo* mutant, the axons in the medial Fas II pathway cross and recross the midline, while the axons in the intermediate and lateral Fas II pathways do not cross the midline (Figure 6A). In contrast, in a *robo;comm* double mutant, the intermediate Fas II pathway is also perturbed and can be seen crossing the midline (Figure 6D). At the very least, this result shows that, in the absence of Robo, Comm still has some additional function that is revealed by removing them both together. Since this additional function affects midline guidance, we speculate that this additional function involves its regulation of Robo2 and/or Robo3. There are several alternative ways in which one might interpret the additional phenotypes seen in the *robo;comm* double mutant. Distinguishing between these models requires having probes for the different subsets of Fas II axons (medial versus intermediate versus lateral); such probes are not yet available, although work is underway to generate these tools (H. Long, personal communication).

Can Comm also downregulate Robo3? It is very difficult for us to do the same experiment as with Robo and Robo2. Both Robo and Robo2 proteins are expressed early in both the CNS and surrounding tissues. Comm can be overexpressed early only in the CNS, and differential reduction of Robo or Robo2 protein in the CNS compared to the surrounding tissue can be assessed (Figures 6C and 6F). However, Robo3 is neither expressed early enough nor in tissues outside the nervous system for a similar comparison. The fact that the *robo, robo2;comm* triple mutant looks like the *robo,robo2* double mutant (in which no axons leave the midline) suggests that if loss of Comm increases the level of Robo3, it does not do so sufficiently to allow any axon to escape the midline. But Robo3 may simply be too weak on its own, even when released from putative Comm downregulation, to repel axons away from the midline.

All of these results and interpretations are further complicated by the existence in the *Drosophila* genome of a gene encoding a second Comm-like protein. Both Comms are all capable when overexpressed of downregulating Robo and Robo2 (T. K.and S. Rajagopalan, unpublished data). How they function to regulate the different Robos is under investigation.

Panneural Expression of Robo2 in a *robo* or *robo2* Mutant Background

A canonical test used to confirm the identity of a gene is to rescue its mutant phenotype by expression of a transgenic copy of the gene. This has been possible in previous studies on Robo because the *robo* gene is normally expressed by all neurons, and, so, the mutant can largely be rescued by expressing the cDNA in all neurons. Unfortunately, this is not possible for *robo2* because its normal pattern of expression is so specific and dynamic, and the gene is too large for standard genomic rescue experiments ($>$40 kb). At present, we know of no promoter elements that would drive cDNA expression initially in all ipsilaterally projecting axons and later in only the neurons whose axons normally extend in lateral pathways. Expression of Robo2 in all neurons or in the subsets for which there are available expression systems (GAL4 drivers) results in gain-of-function phenotypes, indicating that the events of axon guidance are exquisitely sensitive to the specific spatial and temporal patterns of Robo2 expression.

Panneural expression of Robo2 also cannot rescue a *robo* mutant, even though panneural expression of Robo can. This result further indicates that Robo and Robo2 are not identical in their output—they cannot simply substitute for one another. When Robo2 is panneurally expressed in a *robo* mutant, the phenotype varies from segment to segment. Some segments look *comm* and others *robo*.

Where and how much Robo2 is expressed is critical to its function. In wild-type embryos, Robo2 is localized to axons in the lateral pathways; when Robo2 is misexpressed on axons that normally extend close to the midline, they steer outward and join more lateral tracts (see Simpson et al., 2000).

In an earlier section, we described that when Robo2 is panneurally increased in a wild-type background, a phenotypic series is generated that ranges from appearing like *robo* at lower levels to like *comm* at higher levels. However, overexpressing Robo2 in all neurons in a *robo2* mutant background causes much more severe disruptions of axon pathfinding. CNS axons are observed leaving the CNS; some of them return into the CNS several segments later. Motor axons in the periphery cross over segment borders and ectopically fasciculate, sometimes with axons from several segments away. The medial, intermediate, and lateral Fas II longitudinal pathways fasciculate together and travel back and forth across the midline repeatedly. This genotype results in a more disorganized axon scaffold than does Robo2 overexpression in a wild-type background.

It appears that supplying a uniform level of Robo2 on all axons has a much more severe phenotype than sim-

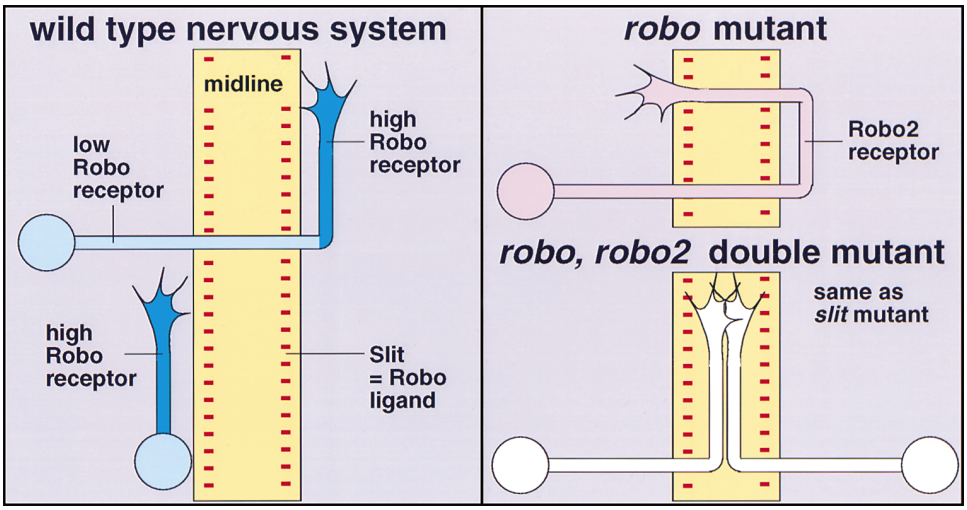

Figure 7. Model for the Role of Robo and Robo2 in the Regulation of Midline Crossing

In *robo* mutants, axons cross the midline but do not remain at the midline, presumably because they are still repelled from the midline due to expression of Robo2. In the absence of both Robo and Robo2, the axons cannot detect the midline repellent Slit and, so, fail to leave the midline, as they do when the repellent Slit is missing (right bottom). Growth cones that do not normally cross the midline express high levels of Robo from the outset, while growth cones that do cross initially express a lower level. After these axons cross once, Robo levels are increased on the contralateral part of the axon to prevent recrossing. In the absence of Robo, some repulsion from the midline is retained because axons still express Robo2. The remaining repulsive force supplied by Robo2 is not sufficient to prevent the ectopic crossing of axons that is seen in a *robo* mutant, but it is enough to stop these axons from lingering at the midline. When both Robo and Robo2 are missing, the axons do not detect Slit and, thus, do not find the midline repulsive.

ply removing all Robo2 or than increasing Robo2 in a wild-type background in which some amount of differential expression is maintained. Thus, the Robo family members appear to have distinct functions, partly mediated by the differences in their sequences and partly by their different spatial and temporal patterns of expression.

Discussion

If commissural growth cones are attracted to Netrin and if the highest concentration of Netrin is at the midline and if growth cones meet their homologs from the other side (for which they have a high affinity) at the midline, then why do these growth cones ever leave the midline? Although we do not yet have a complete answer to this question, we now know that the answer involves both qualitative and quantitative differences between Robo and Robo2 in their repulsive responses to Slit. For growth cones near the midline that do not cross it, Slit forms a strong repulsive barrier. This function is mediated largely by Robo. But for growth cones that do cross the midline, Slit cannot be such a strong repellent, but, rather, it functions in a more subtle fashion, somehow preventing them from lingering at the midline and driving them to cross it. In the absence of Robo, 100% of Robo2 is sufficient for this function but 50% of Robo2 is not. In the absence of Robo2, this function can also be mediated by reduced levels (i.e., 50%) of Robo.

Several years ago, when we discovered that Slit is the ligand for Robo, we were struck by the differences in their phenotypes (Kidd et al., 1999). In the absence of Robo, certain growth cones cross and recross the midline. In contrast, in the absence of Slit, growth cones enter the midline but do not leave it, extending in a single fused longitudinal tract at the midline. Two inferences were drawn from these observations. First, there must be at least one additional Slit receptor that controls midline guidance. In the present paper, we have shown that the *Drosophila* genome encodes three Robo family members, that Robo2 is also a Slit receptor, and that Robo2 functions in midline guidance. The *robo,robo2* double mutant looks just like the *slit* mutant in which all axons project to the midline but do not leave it, suggesting that the functions of these two receptors together can account for all of the functions of Slit in controlling midline guidance (summarized in Figure 7).

Second, because Slit appears to have two different functions in midline guidance (one as a midline repulsive barrier and the second as a midline antilinger signal), it follows that either Robo2 signals differently from Robo (i.e., a qualitative difference in output) or, alternatively, that the low levels of Robo2 (i.e., a quantitative difference in output) on growth cones crossing the midline give rise to the perceived qualitative difference in growth cone behavior. The paper describing the discovery of Slit as the Robo ligand (Kidd et al., 1999) ended with the following statement:

> Whether we are dealing with two qualitatively different negative responses or, alternatively, quantitative differences in a common repulsive mechanism is not yet clear. Teasing this mystery apart in the future should shed some light on how growth cones make stereotyped and divergent decisions at complex choice points.

The results presented in the present paper lead us to conclude that there are indeed both qualitative and quantitative differences between Robo2 and Robo and that each receptor plays a unique role in the control of

midline guidance. First, they have different sequences. *Drosophila* Robo shares four conserved motifs in its cytoplasmic domain with Robo receptors in mammals (Kidd et al., 1998a; Bashaw et al., 2000). Robo2 and Robo3 contain the first two conserved sequences (CC0 and CC1) but are missing the second two (CC2 and CC3), including the Enabled binding site (Bashaw et al., 2000).

Second, when overexpressed panneurally, Robo and Robo2 generate different phenotypic series. Increasing levels of Robo lead to an increasing strong commissureless phenotype in which axons do not cross the midline. In contrast, increasing levels of Robo2 leads to a biphasic phenotypic series in which, at lower levels, too many axons cross the midline (i.e., the embryo looks like *robo*), while, at higher levels, too few axons cross the midline (i.e., the embryo looks like *comm*). This difference suggests that the outputs of Robo and Robo2 are different and that moderate levels of Robo2 can interfere with or decrease the output of Robo, possibly by heterodimerizing with it. Nevertheless, Robo2 has its own repulsive output, and, at higher levels of expression, can drive all axons away from Slit at the midline.

Third, the two receptors cannot functionally substitute for one another. Panneural expression of Robo can largely rescue a *robo* mutant phenotype, whereas panneural expression of Robo2 cannot but, rather, leads to new phenotypes.

What does Robo3 do? Robo and Robo2 play additional roles beyond the decision of whether to cross or not to cross the midline. Robo3 is involved in this second function. The patterns of expression of Robo2 and Robo3 suggest a role in the specification of lateral position (see Simpson et al., 2000). Robo and Robo2 are expressed earlier than Robo3. By the time Robo3 appears, it is highly restricted to primarily those neurons whose axons project in intermediate and lateral pathways. Robo remains expressed by all neurons, although the protein is highly restricted to the longitudinal and not the commissural segments of their axons.

Robo2 is the most dynamic in terms of its pattern of expression. Early during axon outgrowth, Robo2 is expressed by many neurons, including all of the neurons whose axons project ipsilaterally to pioneer the first longitudinal axon pathways, including pCC, vMP2, MP1, and dMP2. This expression correlates with its role in midline guidance. But about the stage that Robo3 begins to appear (late stage 13 to early stage 14), the pattern of expression of Robo2 begins to become much more restricted. Robo2 disappears from pCC, vMP2, MP1, and dMP2. Instead, it begins to be expressed in the restricted subset of neurons whose axons project in lateral pathways. These patterns of expression of Robo2 and Robo3 are striking and suggest a role in controlling lateral position in the developing CNS. In the next paper, we use loss-of-function and gain-of-function genetic analysis to show that the combinatorial code of Robo receptors does indeed control lateral position.

Experimental Procedures

Molecular Biology

The LD λZap phage library (Rubin et al., 2000) was screened using several probes generated by PCR to obtain *robo2* and *robo3* cDNAs; ~1 million clones were plated for each screen. Independent *robo2* and *robo3* clones were sequenced on ALF and ABI machines and compared to existing *Drosophila* sequences using BDGP Blast and MegAlign (Lasergene). PCR and sequencing primers were ordered from Genosys. cDNAs were subcloned from pBluescript (Stratagene) into the pUAST *Drosophila* transformation vector, pCite, and pGEX vectors for in vitro binding studies and pQE vectors for antigen generation using standard cloning procedures. NotI and XhoI restriction sites were introduced by PCR to allow the cloning of *robo2* without UTRs into pUAST. Clades and phylogenetic trees were constructed using the PAM250 weight matrix and the Clustal alignment algorithm. The position of the *robo2* EP2582 insert was determined by plasmid rescue and inverse PCR. The extent of the *robo2* deficiencies was determined by sequencing the PCR product generated by primers surrounding the deletion site.

RNA Localization and Protein Immunocytochemistry

For RNA in situ analysis, probes of ~1 kb from both ends of the cDNAs were used and gave similar results (procedure described in Tear et al., 1996). Immunocytochemistry was done as previously described (see Patel, 1994; Kidd et al., 1998a, 1998b) with the following modifications: all staining using antibodies to Robo, Robo2, and Robo3 were performed on fresh, nonmethanol-stored embryos, using the detergent Tween 20 rather than Triton X-100. Connectin staining was performed according to Kidd et al. (1998a), with amplification using Vectastain Elite ABC kit. For HRP staining, the following concentrations were used: Robo mAb 13C9, 1:10; Robo2 polyclonals, 1:500 to 1:1000; Robo3 mAb 14C9, 1:10; 1D4, 1:5; BP102, 1:10; and Connectin mAb C1.427 (gift from R. White), 1:10. Secondary antibodies were obtained from Jackson Labs.

Protein–Protein Interactions

In vitro translation and binding to GST beads was performed as described in Bashaw et al. (2000). To generate AP-RoboEcto and AP-Robo2Ecto, cloning sites were introduced into *robo* and *robo2* by PCR immediately after the signal sequence and before the transmembrane domain. These fragments were cloned into pSecTag (Invitrogen) with the alkaline phosphatase coding sequence upstream. 293 cells were transfected using Fugene 6 (Roche Molecular Biochemicals), and stable lines were generated by selection with Zeocin. Equilibrium binding was performed essentially as described by Cheng and Flanagan (1994). COS cells were transfected with pcDNA3-slit using Fugene 6. After 48 hr, the cells were rinsed with HBHA buffer and incubated with various concentrations of AP fusion proteins for 90 min at room temperature. Cells were rinsed six times with HBHA buffer, lysed with 1% Triton X-100 and 10 mM Tris (pH 8.0), and heat inactivated at 65°C for 15 min. Samples were cooled to room temperature and mixed with an equal volume of SEAP buffer. Absorbance was measured at 405 nm. Binding curves were fitted using the Hill equation.

Genetics and Fly Stocks

EP and GAL4 lines were obtained from the Berkeley *Drosophila* Genome Project, and deficiencies covering the *robo2* region Df(2L) 45120, 45150, and 45500 were ordered from the Umea Stock Center. *elav-GAL4 3A* and *3E* were generated by Aaron DiAntonio by mobilizing the C155 *elav-GAL4* enhancer trap insert. *enabled* alleles *GC1* and *GC5*, *slit* alleles *1* and *2*, *robo* alleles *z1772*, *GA285*, and *z570*, and *comm* alleles *Δe39* are all published reagents, as are the driver stocks *ScaGAL4* and the transgenes *UAS-comm* and *UAS-robo* (Kidd et al., 1998a, 1998b). Stocks were made using CyOWgβgal and TM6UBXβgal balancers for experiments in which counterstaining to confirm genotype was desirable. Recombinant chromosomes, imprecise excisions, and transformants were generated by standard techniques.

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GenBank Accession Numbers

GenBank accession numbers for *D-robo2* and *D-robo3* are AF312579 and AF312580.